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Ultrastructure and Chemical Composition of the Sheath of *Leptothrix discophora* SP-6

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Light microscopy and transmission electron microscopy of thin sections and metal-shadowed specimens showed that the sheath of *Leptothrix discophora* SP-6 (ATCC 51168) is a tube-like extracellular polymeric structure consisting of a condensed fabric of 6.5-nm-diameter fibrils underlying a more diffuse outer capsular layer. In thin sections, outer membrane bridges seen to contact the inner sheath layer suggested that the sheath fabric was attached to the outer layer of the gram-negative cell wall. The capsular polymers showed an affinity for cationic colloidal iron and polycationic ferritin, indicating that they carry a negative charge. Cell-free sheaths were isolated by treatment with a mixture of lysozyme, EDTA, and *N*-lauroylsarcosine (Sarkosyl) or sodium dodecyl sulfate (SDS). Both Sarkosyl- and SDS-isolated sheaths were indistinguishable in microscopic appearance. However, the Mn-oxidizing activity of Sarkosyl-isolated sheaths was more stable than that of SDS-isolated sheaths. The Sarkosyl-isolated sheaths also contained more 2-keto-3-deoxyoctanoic acid and more outer membrane protein than SDS-isolated sheaths. The oven-dried mass of detergent-isolated sheaths represented approximately 9% of the total oven-dried biomass of SP-6 cultures; the oven-dried sheaths contained 38% C, 6.9% N, 6% H, and 2.1% S and approximately 34 to 35% carbohydrate (polysaccharide), 23 to 25% protein, 8% lipid, and 4% inorganic ash. Gas-liquid chromatography showed that the polysaccharide was an approximately 1:1 mixture of uronic acids (glucuronic, galacturonic, and mannuronic acids and at least one other unidentified uronic acid) and an amino sugar (galactosamine). Neutral sugars were not detected. Amino acid analysis showed that sheath proteins were enriched in cysteine (6 mol%). The cysteine residues in the sheath proteins probably provide sulfhydryls for disulfide bonds that play an important role in maintaining the structural integrity of the sheath (D. Emerson and W. C. Ghiorse, J. Bacteriol. 175:7819–7827, 1993).

Leptothrix discophora is a member of the *Sphaerotilus-Leptothrix* group of sheathed bacteria (38, 55). Bacteria in this group are abundant in iron- and manganese-rich redox-gradient environments such as swamps, iron seeps, and springs. They also cause severe problems with biofouling and clogging in water distribution and drainage systems. *Leptothrix* spp. often are identified and classified in natural samples by the microscopic appearance of their iron- and manganese oxide-encrusted sheaths (26). Because of their ability to bind and oxidize iron and manganese extracellularly, and because of the binding of trace metals to the iron and manganese oxides, these bacteria are attractive candidates for use in model exopolymer-based metal-binding biofilm metal recovery systems (24, 36, 39).

Leptothrix spp. are similar in physiology and cellular structure to closely related gram-negative aerobic heterotrophic pseudomonads (1, 2, 23, 55). Sheath formation and extracellular metal-oxidizing ability are the two major phenotypic criteria distinguishing them from closely related bacteria. The manganese- and iron-oxidizing activities of *L. discophora* SS-1 (ATCC 43182) are at least partly determined by metal-oxidizing proteins excreted by the cells in association with extracellular polymers (3, 10, 13). Presumably the excreted metal-oxidizing proteins are associated with polymers located in the sheath. However, strain SS-1 irreversibly lost the ability to form a sheath soon after it was isolated (2). Recently we reported the isolation and maintenance of a new strain of *L. discophora*, SP-6 (ATCC 51168), which can maintain its sheath-forming ability in laboratory cultures under slow-growth conditions (19). A sheathless variant, strain SP-6(sl) (ATCC 51169), also was isolated. Taxonomic studies (19, 57) showed that strains SP-6 and SP-6(sl) are very similar but not identical to strain SS-1.

The importance of the sheath for survival of *Leptothrix* and *Sphaerotilus* cells in their native habitats cannot be overstated. However, the exact ecological functions of the sheath are not well understood. It is known that the sheath of *L. discophora* is the site of ferromanganese oxide deposition (19) which may protect the cells inside from environmental stresses such as high concentrations of toxic metal cations or toxic oxygen species (23). Frequent microscopic examinations of the surface film of the wetland from which strain SS-1 was originally isolated (25) have shown that *Leptothrix* sp. cells inside sheaths are protected from grazing by protozoa (17). Other probable advantageous functions of the sheath such as accumulation of nutrients, attachment, and regulation of positioning in the environment indicate that the sheath, like many other biological structures, provides multiple benefits for this organism's survival in nature.

Previous work has identified a general pattern in the structure and chemistry of the sheaths of *Sphaerotilus natans* (22, 30, 45) and a few filamentous cyanobacteria (4, 31, 43, 46, 47, 56). These bacterial sheaths consist of a meshwork of polysaccharide- and protein-rich fibrils. Until now, no similar work has been published concerning the sheaths of *Leptothrix* spp.

Here we confirm that the ultrastructure and chemical composition of intact cellular filaments and cell-free sheaths of *L. discophora* SP-6 are similar to those of *S. natans*. We also show that the sheath fabric consists of a matrix of anionic heteropolysaccharides and proteins associated with the outer membrane of the gram-negative wall. Finally, we establish that

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sheath proteins contain a high relative abundance of cysteine residues which provide sulfhydryls for the disulfide bonding responsible for maintaining the structural integrity of the sheath (20). (A preliminary report of this work was presented previously [18].)

MATERIALS AND METHODS

Bacterial cultures and growth conditions. The isolation and maintenance of *L. discophora* SP-6 (ATCC 51168) were described previously (19). For the studies described here, the cells were grown statically in mineral salts-vitamins-pyruvate (MSVP) medium as previously described (19). Culture volumes ranged from 100 ml contained in 250-ml Erlenmeyer flasks up to 16 liters contained in 20-liter carboys. Culture volumes up to 48 liters were obtained by pooling the contents of four 16-liter cultures. Cultures larger than 100 ml were aerated by gently bubbling glass wool-filtered air (two to four bubbles per second) through a sterile 3.0-mm-inside-diameter glass tube inserted through a rubber stopper to the bottom of the flask. Cultures were incubated to the late log phase of growth for 40 to 48 h in a walk-in incubator at 25°C.

Lysozyme-EDTA-detergent lysis isolation procedure. The protocol used for isolating cell-free sheaths was based on the lysis procedure described by Romano and Peloquin (45) for *S. natans*. SP-6 was grown in MSVP liquid medium to an oven-dried (see below) biomass density of approximately 0.15 mg/ml (A_{600} = 0.20) and harvested by centrifugation (8,000 × *g*, 8 min, 10°C). The pellet was resuspended in deionized water to a density 20 times more concentrated than the original. This suspension was then homogenized by passing it repeatedly through a 25-gauge needle on a 10- or 50-ml syringe for small volumes (<50 ml) or through a 14-gauge cannula for larger volumes to disrupt large clumps of filaments that formed in the cultures. Lysozyme and EDTA (both from Sigma Chemical Co., St. Louis, Mo.) were then added to the homogenized suspension to final concentrations of 150 μg/ml and 2.5 mM, respectively. This mixture was incubated at 37°C for 30 min. Next, a detergent, either *N*-lauroylsarcosine (Sarkosyl) or sodium dodecyl sulfate (SDS), was added to the suspension to achieve a final concentration of 1% (wt/vol). The detergent mixture was incubated for 20 min at room temperature (approximately 23°C). Microscopic observations showed that cells were lysed and cellular debris was removed (19) from the sheath. The empty sheaths were washed four times by centrifugation, with resuspension each time in deionized H_2O to the same volume as that used in the lysis mixture. After the final centrifugation, the sheath pellet was resuspended to one-fifth the wash volume to achieve a 100-fold concentration of the original sheath suspension. The concentrated suspension was stored in deionized H_2O at 0°C for up to 10 days until it was used for ultrastructural or chemical analysis. This protocol proved successful for isolating cell-free sheaths from culture volumes ranging from 100 ml up to 48 liters.

Light microscopy. A Zeiss standard 18 microscope equipped with 40× (numerical aperture = 1.0) and 100× (numerical aperture = 1.3) oil immersion phase contrast lenses was employed to determine the presence of sheath structures on cells and to evaluate the purity of the sheaths after isolation. Visualization of sheaths was enhanced by staining with cationic colloidal iron stain as described previously (19, 48). Light photomicrographs were recorded on Kodak Ektachrome T-160 color film with a Zeiss MC63 35-mm camera system.

Metal shadowing and negative staining. Cultures were grown in liquid MSVP medium for 40 to 48 h and harvested by centrifugation, and sheaths were isolated as described above. Samples of the original culture and the isolated sheaths were fixed, sectioned, and poststained for transmission electron microscopy (TEM) as described previously (19). For metal shadowing, isolated sheaths suspended in deionized water were mounted on Formvar-coated copper electron microscope grids by floating the grids film side down on a drop of the sheath suspension for 2 min. The grids were blotted with filter paper and allowed to dry in air. The grids were coated in a Balzers BAE 080 vacuum evaporator with Pt-Pd (80:20) at a fixed angle of 20° from the grid surface. For negative staining, the grids carrying air-dried sheaths were floated face down on a drop of either 2% ammonium molybdate, pH 7.0; 2% potassium phosphotungstic acid, pH 7.0; or 2% aqueous uranyl acetate, pH 4.5. After 15 s, the grids were removed, blotted, and allowed to dry in air.

Labeling with PCF. Polycationic ferritin (PCF) (Electron Microscopy Sciences, Fort Washington, Pa.) was used to label anionic sites on sheaths by centrifuging 1.0 ml of SDS-isolated sheath suspension (0.7 mg [dry weight]/ml) at 8,800 × *g* for 6 min in an Eppendorf Microfuge. The pellet was resuspended in 0.5 ml of 0.1 M sodium cacodylate buffer, pH 7.0, containing 10 mM $CaCl_2$. A 0.1-ml suspension containing 100 mg of PCF obtained from the supplier was diluted to 0.5 ml with the cacodylate buffer; 0.25 ml of the diluted suspension was mixed with 0.5 ml of the sheath suspension, and the mixture was incubated for 10 min at room temperature. The mixture was then centrifuged, and the pellet was washed once by centrifugation in deionized H_2O. The washed pellet was resuspended in 1.0 ml of the cacodylate buffer containing 1% (vol/vol) glutaraldehyde (final concentration). PCF-labeled sheaths were embedded in Epon-Araldite resin for thin sections as described previously (19).

Sheaths on cellular filaments were labeled with PCF by centrifuging 6 ml of a 3-day-old MSVP culture (A_{600}, ~0.18), washing the pelleted material by centrifugation once in deionized H_2O, and resuspending the final pellet in 0.5 ml of the cacodylate buffer. PCF labeling, glutaraldehyde fixation, and preparation for TEM thin sections were as described above.

As a negative control for PCF labeling, isolated sheaths were treated with ethyldimethyl-aminopropyl carbodiimide (EDC) (Sigma) in the presence of glycine methyl ester (GME) (Sigma) prior to PCF treatment to covalently modify and neutralize carboxylate groups (12). In this procedure, 2.5 ml of sheath suspension was combined with 2.5 ml of 4 M GME. The pH of the mixture was adjusted to 4.75 with NaOH; 200 mg of crystalline EDC was then added to the mixture while it was mixed with a magnetic stir bar. The pH was held at 4.75 by addition of either NaOH or HCl. After 1 h of incubation at room temperature, the reaction was stopped by the addition of 1.5 ml of 1 M acetate buffer, pH 4.75. The EDC-treated sheaths were washed four times in 16 ml of deionized H_2O and then labeled with PCF, fixed, and prepared for thin sections as described above.

TEM. All grids were examined in a Philips 300 transmission electron microscope at an accelerating voltage of 80 kV and instrument magnifications up to ×70,000. Electron photomicrographs were recorded on Kodak electron image film.

Dry weight determinations. Dry weights were determined by placing 1.0-ml duplicate or triplicate aliquots of washed culture material or isolated sheaths suspended in deionized H_2O in tared aluminum pans and drying the samples to constant weight at 90°C. The pans were weighed on a Mettler analytical balance, and the dry weight was calculated by difference. The amount of water in hydrated sheaths was estimated by centrifuging a suspension of isolated sheaths (10,000 × *g*, 10 min, 10°C) in deionized water. Samples of the concentrated pellet

were weighed in an aluminum pan before and after drying at 90°C. The loss in mass was taken as an estimate of the free-water content of the sheath.

Carbohydrate analyses. The reducing-sugar content of isolated sheaths was determined by the cupric reduction technique with glucose as a standard (51). D-Glucose analysis was done by hydrolyzing 4.5 mg (dry weight) of washed sheath material in 4 N HCl (final concentration) for 45 min at 100°C. The hydrolysate was dried under a stream of N_2 at 40°C, and D-glucose was assayed enzymatically by using a glucose oxidase-horseradish peroxidase clinical assay kit (Sigma). Sialic acid was assayed by the resorcinol method (51) with *N*-acetylneuraminic acid (Sigma) as a standard. Uronic acid content was determined by the method of Blumenkrantz and Asbøe-Hansen (8) with galacturonic acid as the standard.

Amino sugars were assayed according to the general procedure described by Boas (9). Hydrated sheath material containing the equivalent of 1 mg (dry weight)/ml was hydrolyzed in 4 N HCl (final concentration) for 6 h at 100°C under an atmosphere of N_2 in a glass vial sealed with a butyl rubber stopper. After hydrolysis, the sample was evaporated to dryness, either under a stream of N_2 at 40°C or in vacuo over NaOH pellets at room temperature. The dried hydrolysate was reconstituted in deionized H_2O to a concentration between 5 and 10 mg (dry weight)/ml and passed through an AG 50W-X8 cation-exchange resin (100 to 200 mesh, H^+ form; Bio-Rad). The cation-exchange column was made by adding 0.5 ml of resin slurry to a Pasteur pipet plugged with glass wool. After the sample was added to the column, it was treated with 12 ml of deionized H_2O to wash through neutral sugars and amino acids. The amino sugars were eluted from the column with 6 ml of 2 N HCl. The acid effluent was dried under a stream of N_2 and then redissolved in deionized H_2O to a concentration equivalent to 2 mg (dry weight)/ml and assayed by the Elson-Morgan method (9) with galactosamine as a standard. In some cases, aliquots of the eluted material were acetylated with 1.5% acetic anhydride (51) and assayed for *N*-acetylhexosamine by the Morgan-Elson procedure (15) with acetylated glucosamine and galactosamine (Sigma) as standards.

Protein and amino acids. Analysis for protein content was done by the microassay procedure in a Bradford-method reagent kit (Bio-Rad) with lysozyme as a standard. The sheath material and standard were both treated with 0.75 N NaOH at 100°C for 5 min before protein analysis.

For analysis of amino acids in washed, isolated sheaths, a suspension containing 1 mg (dry weight) of the sheath material was hydrolyzed for 1 h in 6 N HCl at 150°C and then analyzed on an amino acid analyzer at the Cornell Biotechnology Institute amino acid analysis facility.

Nucleic acids. Analysis of total nucleic acids was done by measuring the A_{260}/A_{280} ratio of isolated sheath suspensions with a Beckman model 25 spectrophotometer (44). DNA in the sheath suspensions was determined by extracting the suspension with cold 10% trichloroacetic acid and then assaying the extract by using a diphenylamine assay (29). RNA content was analyzed by extracting detergent-isolated sheaths with cold perchloric acid and then assaying the extract by the orcinol procedure (29).

Lipids. Isolated sheath suspensions (100 mg [dry weight]) were extracted with chloroform-methanol-water (2:1:0.8) in a separatory funnel according to the procedure of Bligh and Dyer (7). The resulting chloroform layer was placed in a tared, sintered glass stoppered bottle (25-ml volume) and dried under a stream of N_2 at 40°C. Prior to being tared, the bottle had been flushed with N_2 and placed in a desiccator at room temperature for 3 h. The bottle containing the dried chloroform residue was placed in a desiccator for 2 to 3 h prior to reweighing of the contents. The difference in dry weight was taken as an estimate of the amount of free lipid in the sheath. Phase contrast light-microscopic examination showed that lipid extraction did not alter the appearance of the sheaths in the aqueous phase. The aqueous-phase material was then washed with deionized H_2O and hydrolyzed in 3 N HCl for 2.5 h at 100°C. The resulting hydrolysate was dried under a stream of N_2 and reextracted with chloroform-methanol-water. The dried residue of the chloroform layer from the second extraction was weighed to determine the amount of lipid released after acid hydrolysis.

Pyruvate, KDO, and PHA. Pyruvate analysis was done by hydrolyzing 5 mg (dry weight) of sheath in 1 N HCl for 2.5 h at 100°C and then neutralizing the hydrolysate with NaOH and assaying it with a lactate dehydrogenase-NADH clinical pyruvate assay kit (Sigma). Analysis of 2-keto-3-deoxyoctonic acid (KDO) (Sigma) an indicator of outer membrane contamination in isolated sheaths, was done by the method of Karkhanis et al. (33), with KDO as a standard. A total membrane fraction from a culture of ensheathed SP-6 cells, prepared as described by Adams and Ghiorse (2), served as a positive control. Poly-β-hydroxyalkanoate (PHA) was assayed by the procedure of Hanson and Phillips (28) with PHA purified from *Bacillus megaterium* (gift of Eugene Delwiche, Cornell University) as a standard.

Elemental analysis and inorganic residues. C, H, N, P, and S analyses were done on isolated sheath suspension by following standard procedures at the Agronomy Analytical Laboratory, Cornell University. C, H, and N were determined by using a Perkin-Elmer model 240C elemental analyzer. Total P was determined by the vanadomolybdophosphoric acid method. Total S was determined turbidimetrically as SO_4^{2-} after magnesium nitrate ashing. Inorganic SO_4^{2-} was determined turbidimetrically (50) on approximately 4 mg (dry weight) of sheath, hydrolyzed for 2 h in 1 N HCl at 100°C and dried in vacuo prior to the assay.

The inorganic residue of washed sheaths was determined by heating 100 mg (dry weight) of a sheath suspension in a tared porcelain crucible at 550°C for 45 min. The crucible was cooled to room temperature in a desiccator and then weighed on a Mettler analytical balance; the noncombustible residue was taken as an estimate of the inorganic content of the sheath.

Identification of amino sugars and uronic acids by GLC. For amino sugars, sheath suspensions containing approximately 12 mg (dry weight) of SDS-isolated sheath material were hydrolyzed in 4 N HCl (final concentration) for 6 h and then dried in vacuo over NaOH pellets at room temperature. The dried material was reconstituted in 1.0 ml of deionized H_2O and neutralized to pH 7 with 1.5 N NaOH. The neutralized sample was reduced with $NaBH_4$, and the free amino sugars were derivatized to their alditol acetates (53). Standards of D-galactosamine (NGal), D-glucosamine (NGlu), and D-mannosamine (NMan) (Sigma) and their corresponding neutral sugars were derivatized and chromatographed under conditions identical to those employed for sheath material.

For analysis of uronic acids, an identical sample of SDS-isolated sheaths was hydrolyzed in 4 N HCl for 90 min, dried in vacuo over NaOH, reconstituted in distilled H_2O, and neutralized as described above for amino sugars. Uronic acids were derivatized to their alditol acetates and chromatographed according to the procedure of Lehrfeld (35). This required reduction of the sample three times with $NaBH_4$ prior to conversion to the alditol acetate. Standards of D-galacturonic acid (GalA), D-glucuronic acid (GluA), and D-mannuronic acid

(ManA) (Sigma), as well as the corresponding neutral sugars, were derivatized simultaneously with the sheath sample.

Amino sugars and uronic acid derivatives were separated by gas-liquid chromatography (GLC) on a Hewlett-Packard 401 high-efficiency gas chromatograph employing temperature programming. For amino sugars, injection was done at 180°C with a 6-min hold followed by a temperature increase of 3°C/min to 240°C with an indefinite hold at the upper limit. For uronic acids, the initial temperature was 195°C with a 3-min hold, and then the temperature was increased 2°C/min to 240°C. The carrier gas was He, regulated at a flow rate of 40 ml/min for amino sugars and 25 ml/min for uronic acids. The injector was set at 240°C, and the detector temperature was 250°C.

SDS-polyacrylamide gel electrophoresis (PAGE). Isolated sheath suspensions (0.8 ml) were concentrated by centrifugation in an Eppendorf Microfuge as described above. The volume of the pellet residue (~0.15 ml) was doubled with a treatment buffer containing 40 mM bicine buffer (pH 8.8), 20 mM dithiothreitol, 4% SDS, 20% glycerol, and 0.01% bromphenol blue. The mixture was heated at 100°C for 75 s and then loaded on a 12.5% polyacrylamide gel containing SDS (3). Electrophoresis and staining with Coomassie blue (R-250) were done as previously described (19).

RESULTS

Sheath isolation. The lysozyme-EDTA-detergent lysis isolation protocol resulted in sheaths that were devoid of cells as indicated by phase contrast microscopy and cationic colloidal iron staining before and after treatment (Fig. 1). There were no differences in the light microscopic appearances of the sheaths isolated with Sarkosyl or SDS as the detergent. However, the Mn-oxidizing activity of the Sarkosyl-isolated sheaths was more reproducible (16). The Sarkosyl-isolated sheaths also contained approximately twice as much KDO as SDS-isolated sheaths did (Table 1), and their content of outer membrane proteins was higher (see below).

Ultrastructure of sheaths before and after isolation. Thin sections of sheaths containing cells before isolation revealed the sheath as a multilayered structure with a discrete, electron-dense layer (30 to 100 nm) covered by a more diffuse capsular layer of variable thickness (19) (Fig. 2A) and outer membrane blebs which were found to contact the inner sheath layer at many points along the cell surface (Fig. 2). The diffuse capsular material showed a strong affinity for PCF, which appeared as a condensed electron-dense layer at its outer extremities (arrows, Fig. 2B).

The ultrastructure of isolated sheaths (Fig. 3A) closely resembled that of the sheaths seen in whole-cell preparations. The inner, electron-dense layer and the more diffuse capsular layer were clearly visible in both SDS- and Sarkosyl-isolated sheaths. Thin sections of Sarkosyl-isolated sheaths showed outer membrane bleb remnants inside the empty lumen of the sheaths (arrows, Fig. 3A). The remnants were common in Sarkosyl-isolated sheaths but were only rarely seen in SDS-isolated sheaths (not illustrated). These outer membrane remnants likely accounted for the higher concentration of KDO (Table 1) and the greater amounts of major outer membrane proteins (see below) in Sarkosyl-isolated sheaths.

TEM of metal-shadowed, air-dried sheaths showed that they were composed of a matrix of structural fibrils (Fig. 3B). Individual fibrils measured 6.5 (±1.8) nm in diameter. They appeared to be integral structural elements of the inner condensed sheath layer. This interpretation was confirmed in later sheath dissociation studies (20). No subunit structure was discerned in the sheath fibrils by metal shadowing (Fig. 3B) or by negative staining with ammonium molybdate, potassium phosphotungstate, or uranyl acetate (not illustrated).

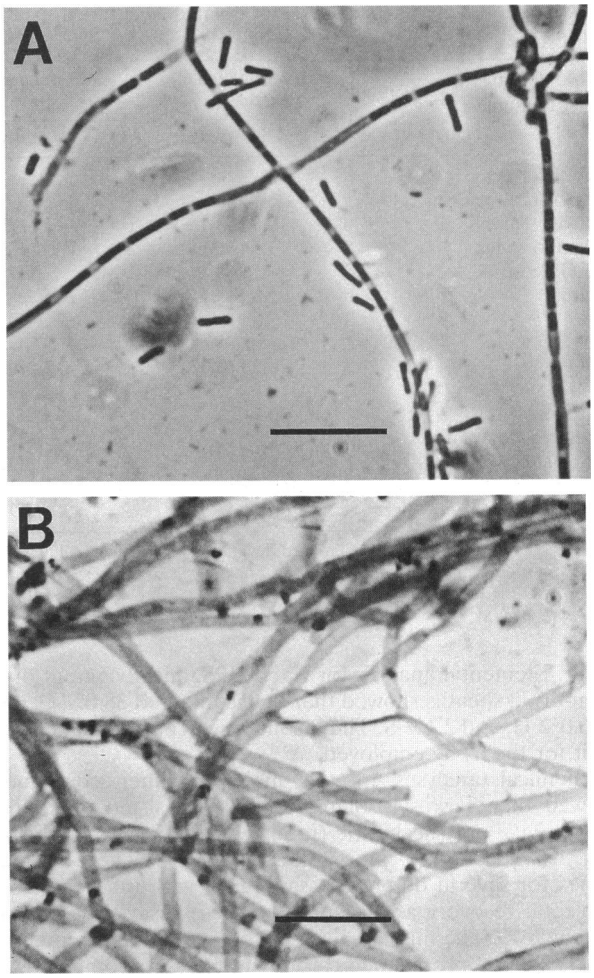

FIG. 1. Phase contrast light micrographs of cationic colloidal iron-stained samples showing ensheathed cellular filaments before (A) and isolated sheaths after (B) treatment by the lysozyme-EDTA-detergent lysis isolation protocol described in Materials and Methods. Both SDS- and Sarkosyl-isolated sheaths appeared the same after treatment and staining with the cationic colloidal iron. Bars = 10 μm.

Thin sections of isolated sheaths labeled with PCF showed the same binding pattern of electron-dense PCF particles as whole filaments (compare Fig. 4A with Fig. 2A). In both cases it appeared that the PCF was bound and precipitated at the extremities of the capsular layer. Little, if any, PCF penetrated to the interior layers of the sheath. When isolated sheaths were pretreated with EDC and GME to modify carboxylate groups prior to PCF labeling, PCF binding was greatly reduced (Fig. 4B). These results indicate that negatively charged carboxyl groups in the sheath capsular layer were responsible for the PCF binding.

Chemical composition of isolated sheath. Dry weight determinations comparing washed oven-dried biomass from cultures containing intact SP-6 filaments with Sarkosyl-isolated sheath material showed that isolated sheaths accounted for approximately 9% of the biomass of the SP-6 filaments. Comparison of wet and dry weights of isolated sheaths showed that the hydrated sheaths consisted of approximately 99%

TABLE 1. Chemical composition of isolated, oven-dried sheaths of *L. discophora* SP-6

Constituent	Sarkosyl-isolated sheaths		SDS-isolated sheaths	
	n	% of dry wt[a]	*n*	% of dry wt
Carbohydrates				
Total reducing sugar	5	33.8 (8.4)	2	34.5 (9.5)
Uronic acid	6	19.8 (3.3)	3	20.7 (4.3)
Amino sugar	5	15.5 (3.3)	2	17.4 (1.8)
D-Glucose	2	<0.1		ND[b]
Protein	6	22.8 (2.8)	3	24.5
Lipid	3	7.8 (1.4)		ND
Ash	2	3.8		ND
KDO	2	0.23 (0.05)	2	0.12 (0.05)
PHA	2	<0.5		ND
DNA and RNA	2	<0.1		ND
Pyruvate	2	<2.0		ND
Sialic acid	1	<1.0		ND
SO_4	2	<0.5		ND
Total recovery		68.5		ND

[a] Numbers in parentheses are standard deviations of the mean value.
[b] ND, not determined.

water. Elemental analysis for C, N, H, S, and P content of the oven-dried sheaths showed that they contained 38.0% C, 6.9% N, 6.0% H, and 2.1% S. The P content was below the detection limit for the assay employed.

Chemical analyses of the major polymer components showed that Sarkosyl-isolated sheaths consisted of approximately 34% polysaccharide (total reducing sugars), 24% protein, 8% lipid, and 4% inorganic ash (Table 1). These are mean values for several different assays, each with inherent errors. The total recovery as measured by these assays was approximately 70%. The missing 30% can be attributed to underestimations in the protein and carbohydrate assays combined with overestimation of the dry weight. The major reducing-sugar components of the sheath polysaccharide were uronic acids (approximately 20%) and amino sugars (approximately 16%). The amino sugars appeared to be in the *N*-acetylated form as indicated by the Morgan-Elson reaction for *N*-acetylhexosamines when *N*-acetylgalactosamine was used as the standard. DNA, RNA, and PHA were below detection limits for the assays employed, indicating that the isolated sheaths were free of these cellular components. Pyruvate, sialic acid, and SO_4, which are commonly reported as constituents of bacterial exopolysaccharides, were not present at detectable levels in the SP-6 sheath. Comparative analyses of SDS-isolated sheaths showed that their total content of reducing sugars, uronic acids, amino sugars, and proteins were all in close agreement with those of Sarkosyl-isolated sheaths; however, the Sarkosyl-isolated sheaths contained approximately twice as much KDO as the SDS-isolated sheaths. As indicated above, the difference in KDO content probably resulted from the greater number of outer membrane blebs seen in electron micrographs of Sarkosyl-isolated sheaths (Fig. 3A and 4B).

The inorganic residue after ashing accounted for nearly 4% of the dry weight of the sheath (Table 1). Presumably, this residue contained mineral elements such as Ca and Mg and traces of other elements from the MSVP medium. No further analysis of inorganic constituents was done.

GLC analysis of the sheath revealed two major components that chromatographed identically to or close to amino sugar standards (Fig. 5A). A peak with a retention time (R_t) of 32.3 min corresponded to NGal. The second peak, with an R_t of 31.0 min, chromatographed close to NMan; however, it was not sufficiently resolved to identify it absolutely. GLC analyses also showed that the uronic acids (ManA, GalA, and GluA) were present in the sheath (Fig. 5B). The R_ts were 18.5, 20.0, and 21.0 min, respectively. There was also an unidentified peak (R_t = 9.5 min) in the uronic acid chromatogram (Fig. 5B). The procedure used for derivatizing uronic acids (35) required three reduction steps with $NaBH_4$ to completely convert the partially reduced lactones of the uronic acids to their fully reduced form. This was necessary for complete conversion to the alditol acetate before chromatography. When only one reduction step was carried out, no peaks were evident in chromatograms of either the sheath or the standards; however, the neutral sugars, mannose, galactose, and glucose, treated with only one reduction chromatographed normally (results not shown). The results of these GLC experiments support the conclusion that the peaks in the sheath chromatogram shown in Fig. 5B were the uronic acids and amino sugars mentioned above. However, absolute identification requires gas chromatography coupled with mass spectrometry, which was not done. Therefore, our GLC identifications must be considered presumptive until gas chromatography-mass spectrometry analyses can be done.

Extraction of Sarkosyl-isolated sheaths with chloroform-methanol-water before and after acid hydrolysis resulted in a total of approximately 7.8% of the dry weight being extracted into the chloroform phase. The total chloroform-extractable material consisted of equal portions of material recovered from sheaths before and after acid hydrolysis. The total chloroform-extractable material was presumed to be lipid (Table 1), although no further analysis for fatty acid composition was done.

SDS-isolated sheaths showed a full complement of common amino acids (Table 2), presumably contained in sheath proteins. The proteins apparently are enriched in cysteine, which accounted for 6 mol% of the total amino acids. Amino acid analyses of Sarkosyl-isolated sheaths closely agreed with those of the SDS-isolated sheaths (results not shown).

Gel electrophoresis. Electrophoretic analysis of isolated sheaths revealed a significant difference between the SDS- and Sarkosyl-isolated sheaths (Fig. 6). Sheaths isolated with Sarkosyl showed three Coomassie blue-staining bands with apparent molecular masses of approximately 89,000, 40,000, and 37,000 kDa (Fig. 6, lane A). These bands correspond closely to three of the major protein bands (88,000, 38,000, and 35,000 kDa) reported in total membrane fractions of both SP-6 and its sheathless derivative, SP-6(sl), under slightly different conditions of electrophoresis (19). These bands were only faintly visible in SDS-isolated sheaths, even though identical amounts of protein were loaded on the gel (Fig. 6). These results confirm the TEM (Fig. 3A and 4B) and KDO analysis (Table 1) results indicating that outer membrane fragments in Sarkosyl-isolated sheaths were effectively removed by isolation with SDS. The absence of protein bands attributable to the sheath shown in Fig. 6 also emphasizes the unusual resistance of the sheath to solubilization by detergents and other chemical treatments known to solubilize proteins (20).

DISCUSSION

Light microscopic and ultrastructural analyses of intact *L. discophora* SP-6 filaments reported here (Fig. 2) and in our other work on sheath structure (19, 20) show that the sheath is composed of a fibrillar matrix of anionic polymers that stain with colloidal iron stain. The sheath is a macromolecular fabric distinct from the outer membrane of the cell wall; however,

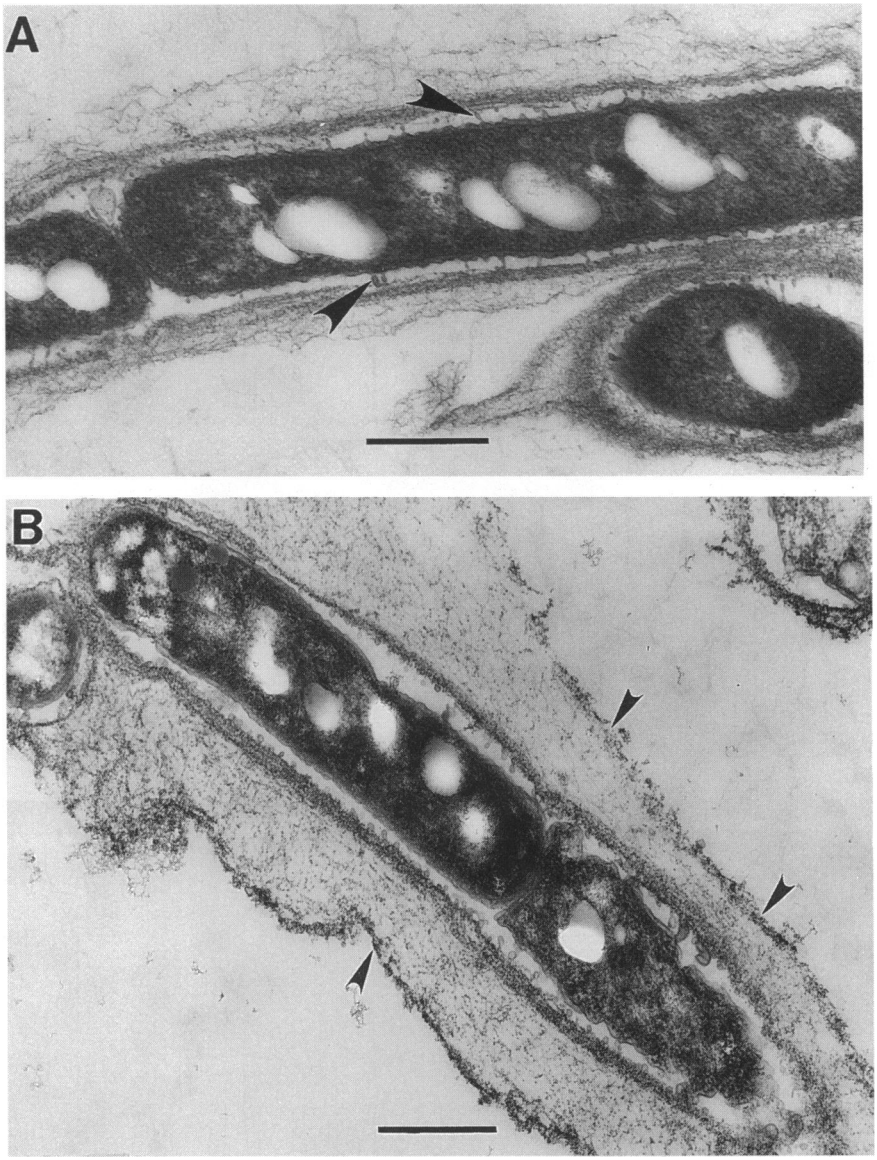

FIG. 2. Electron micrographs of thin sections of *L. discophora* SP-6 filaments fixed by a modified Rytér-Kellenberger procedure as reported previously (19). (A) Longitudinal section showing the sheath structure of untreated cells with the inner and outer layers of the sheath. Note that the blebs arising from the outer membrane (arrows) appear to form bridges to the inner layer of the sheath. (B) PCF-treated filaments showing the outer electron-dense layer (arrows) containing PCF bound to the outer part of the sheath capsular layer. Bars = 0.5 μm.

there appear to be numerous contact points between the cell wall and the sheath that become visible as outer membrane bridges or blebs when cells fixed by the modified Rytér-Kellenberger procedure shrink during fixation and dehydration. As discussed previously (19), the membranous blebs commonly seen in thin sections of whole cells probably are artifacts of the Rytér-Kellenberger fixation and dehydration procedure. However, they may represent points of contact and transport between the sheath and the cell wall. Similar blebs were seen by TEM in thin sections of *L. discophora* SS-1 (2) as well as in cells observed by TEM in natural samples (23, 49). Chemical analyses of isolated sheaths also lead to this conclusion. SDS-isolated sheaths contained less of the outer membrane marker, KDO, and less of three major membrane protein bands seen on SDS-PAGE gels than Sarkosyl-isolated sheaths. SDS is known to solubilize the outer membrane components of gram-negative bacteria more efficiently than Sarkosyl (21). Because the total protein content of sheaths was nearly the same in both SDS- and Sarkosyl-isolated sheaths, we conclude that outer membrane fragments were minor components of the isolated sheaths. SDS removed them more efficiently than did Sarkosyl. Electron micrographs of SP-6 cells fixed by freeze substitution (6, 27) do not show the outer membrane blebs, probably because cellular shrinkage is reduced in freeze-substituted specimens.

It is important to note that Sarkosyl-isolated sheaths, while not as free of outer membrane components as SDS-isolated sheaths, consistently maintained their capacity to oxidize Mn^{2+}. The SDS-isolated sheaths showed variable Mn-oxidizing activity (16). Presumably, the Mn-oxidizing activity, which has been shown to depend, at least partly, on an SDS-stable protein (3), was retained more consistently by Sarkosyl-iso-

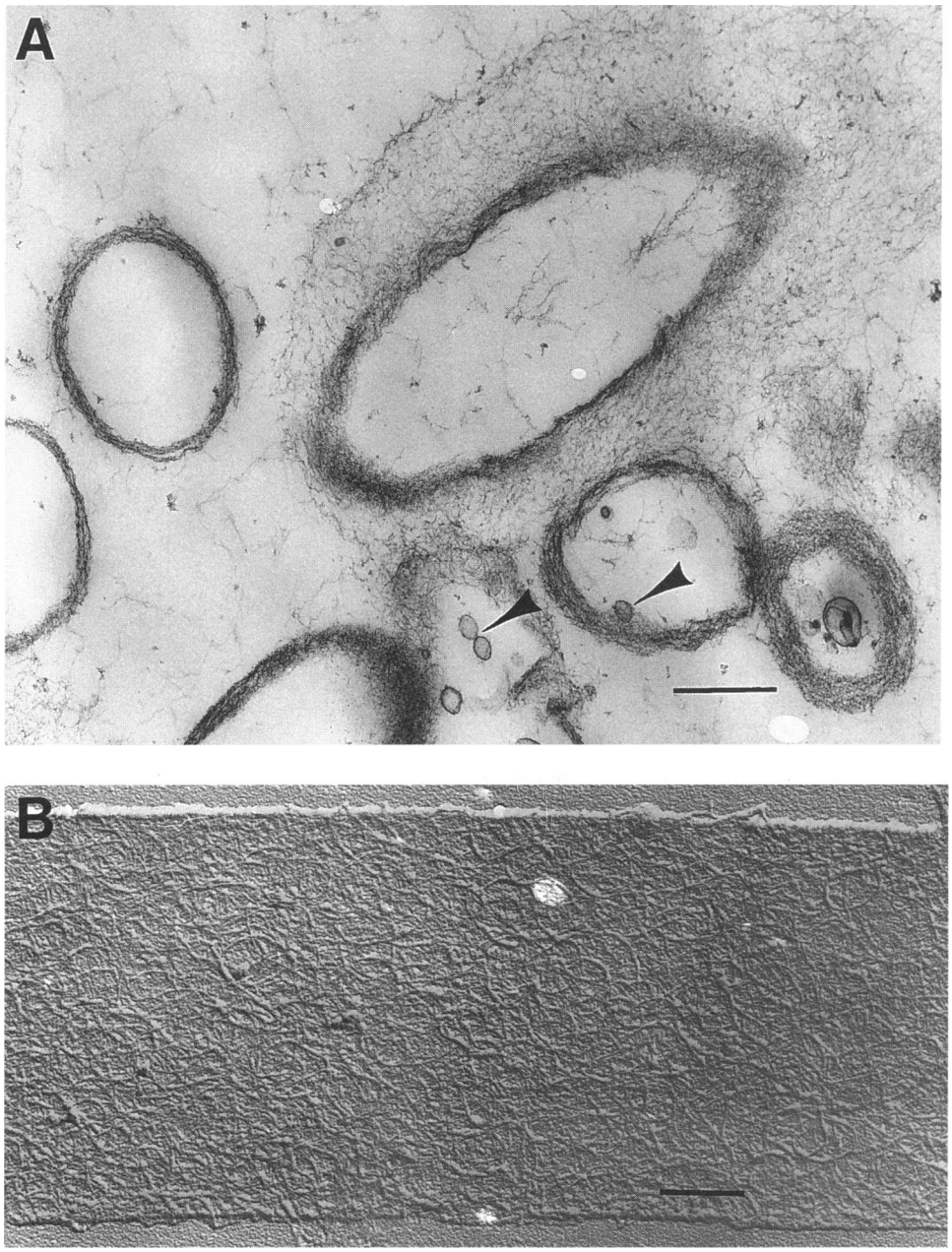

FIG. 3. Electron micrographs of sheaths isolated with Sarkosyl. (A) Thin section showing typical layered sheath structures, some of which contain the remnants of outer membrane blebs (arrows) inside the empty lumen. Bleb remnants were rare in SDS-isolated material. (B) Metal-shadowed (Pt-Pd, 80:20) air-dried sheaths showing the fibrillar appearance of the sheath surface. Bars = 0.5 μm.

lated sheath polymers. This could be due to the fact that SDS is a more potent protein-solubilizing agent than Sarkosyl (21) and thus would be more likely to remove the Mn-oxidizing protein(s) from the sheath during isolation. This is in keeping with our hypothesis that the Mn-oxidizing protein(s) is not an integral component of the sheath polysaccharide fibrils but probably is associated with them in a noncovalent manner (19). An alternative explanation for the loss of Mn-oxidizing activity is that it is inhibited in the presence of SDS (3). The variable activity of SDS-isolated sheaths might then be explained by inefficient removal of SDS during washing.

Our ultrastructural analyses of thin-sectioned and metal-shadowed sheaths clearly show two distinct layers, a diffuse exterior capsular layer of loosely arrayed capsular fibrils (Fig. 2) and a condensed interior layer of tightly associated fibrils (Fig. 3). It is not known whether the inner and outer layers identified by TEM are chemically different. The outer capsular layer could be composed of polymers similar to those reported in *S. natans* by Gaudy and Wolfe (22). The *S. natans* capsular polymers contained uronic acids, whereas the isolated sheaths described by Romano and Peloquin (45) did not. Although our experiments did not address the chemistry of the two different layers, it seems likely that the capsular layer of SP-6 is excreted through the condensed inner sheath layer. Indeed, the micro-

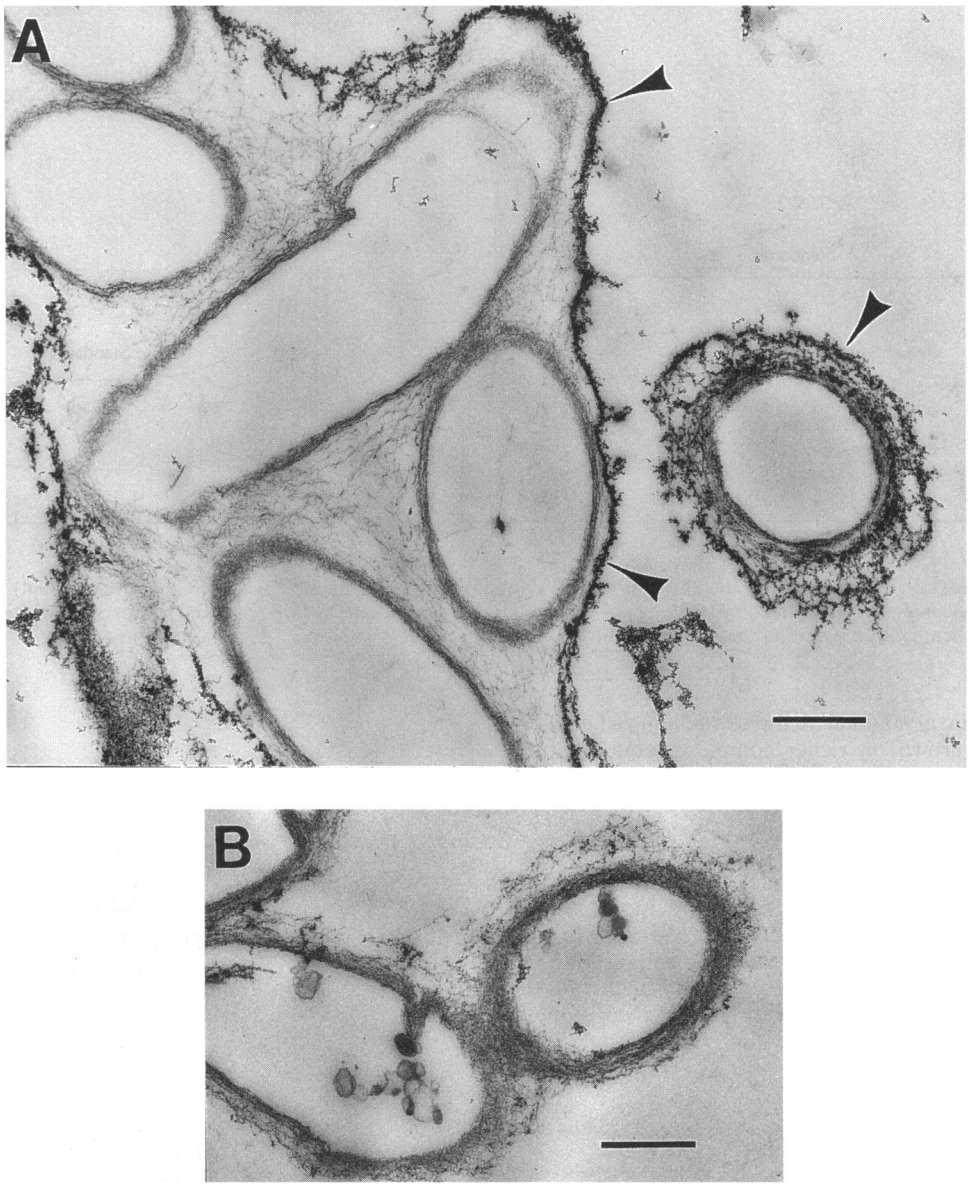

FIG. 4. Electron micrographs of sheath isolated with Sarkosyl. (A) Sheath labeled with PCF. Note the dense concentration of PCF label (arrows) on the outer extremities of the sheath capsular layer. (B) Sheath pretreated with EDC and GME prior to PCF treatment. Note the absence of PCF when carboxylate groups were neutralized prior to PCF labeling. Bars = 0.5 μm.

graphs of freeze-substituted SP-6 filaments (6, 27) clearly show a well-preserved fuzzy extracellular polymer layer on SP-6 cells inside the sheath which appears to contact the inside surface of the overlying condensed sheath layer (see Fig. 5 in reference 27). Whether this material represents a true capsule or long-chain lipopolysaccharide, which has been shown to be preserved by freeze substitution (34), is unknown.

Superficially, the sheath of SP-6 is structurally and chemically similar to sheaths of *S. natans* (30, 45) and cyanobacteria (4, 31, 43, 47, 56). The sheaths of these bacteria have all been reported to be composed of a random meshwork of fibrils that are rich in carbohydrate with lesser amounts of protein. No ordered subunit structures have been described. This is in contrast to the archaea, *Methanothrix* and *Methanospirillum* spp., which possess proteinaceous sheaths with distinctive subunit structures (40, 52).

The gross chemical composition of the SP-6 sheath was similar to that of the *S. natans* sheath. The total carbohydrate, protein, and lipid contents of the SP-6 sheath were 34, 23, and 8%, respectively, compared with 36, 28, and 5.2%, respectively, for *S. natans* (45). However, the polysaccharide constituents differed markedly. The sheath of *S. natans* consisted primarily of neutral sugar, largely glucose, with lesser amounts of glucosamine, and no uronic acids were found (45). In contrast, the SP-6 sheaths consisted of uronic acids and amino sugars, and neutral sugars were not detected. This may be a significant phenotypic difference between the two closely related genera (see reference 14 for a discussion of the phylogeny of *L. discophora* and *S. natans*). On the other hand, it is possible that the differences reflect differences in growth conditions, which have been shown to affect the composition of extracellular polysaccharides of other bacteria (11, 54). In our experiments,

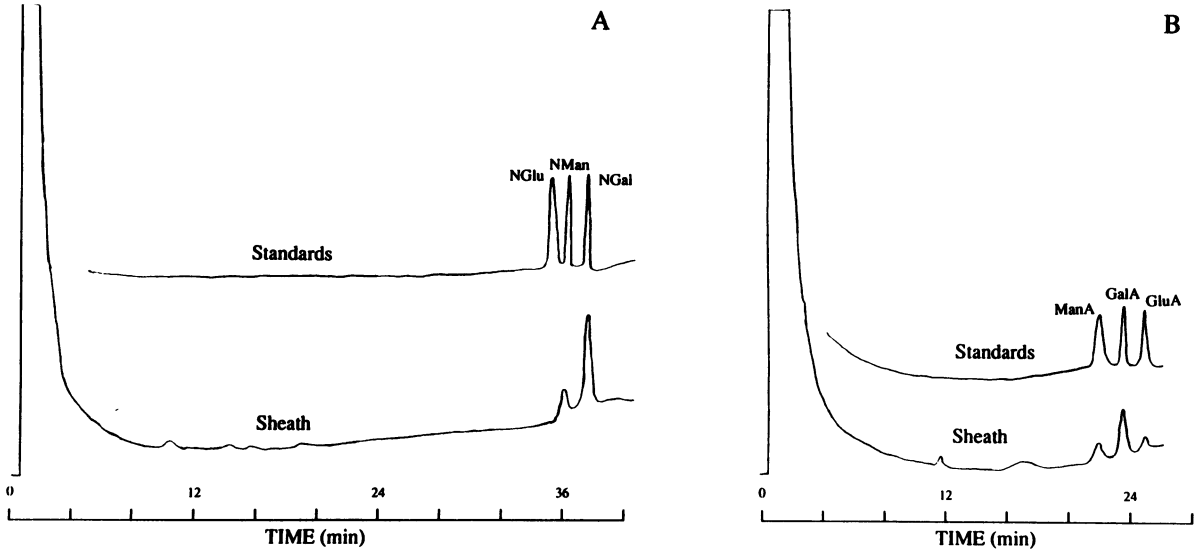

FIG. 5. GLC traces of Sarkosyl-isolated sheaths showing the chromatographs for amino sugars (NGlu, NMan, NGal) (A) and uronic acid (ManA, GalA, GluA) standards (B) with comparable peaks in sheath preparations were hydrolyzed and derivatized in identical fashion; the large initial peaks in the sheath chromatographs are from the solvent, pyridine.

SP-6 was grown in a minimal defined medium, whereas *S. natans* usually is grown on richer complex media. Cultural variability might also account for the variability in uronic acid content of *S. natans* reported by Gaudy and Wolfe (22) and Romano and Peloquin (45) as described above.

From our GLC analyses, we can conclude that the glycan component of the sheath is a complex heteropolysaccharide that has a high cationic metal-binding affinity due to the net negative charge imparted by the uronic acid residues (32, 37). The sheath clearly plays a role in the binding and oxidation of Mn^{2+} and probably Fe^{2+} as well (19). It seems likely that the uronic acid carboxyl groups are primary sites of divalent metal binding in the sheath. The role of the amino sugar is not clearly

TABLE 2. Amino acid composition of SDS-isolated sheaths of *L. discophora* SP-6[a]

Amino acid	Abundance (mol%)
Gly	31
Glx[b]	12
Asx[c]	8
Ser	8
Ala	7
Cys[d]	6
Leu	5
Val	5
Thr	4
Pro	3
Arg	3
Ile	2
Phe	1
Met	1
Lys	1
Tyr	1
His	1

[a] The abundance of Trp was not determined.
[b] Glutamine or glutamate.
[c] Asparagine or aspartate.
[d] Note the relatively high abundance of cysteine at 6 mol%.

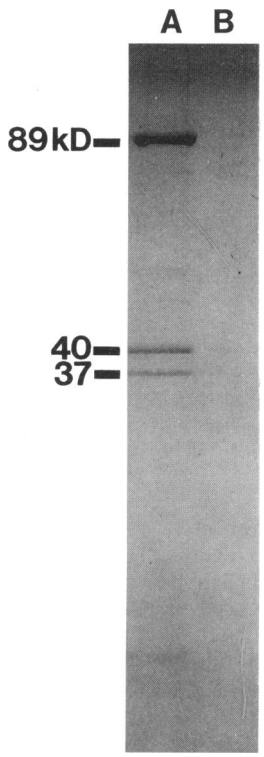

FIG. 6. SDS-PAGE electrophoretogram comparing proteins in the sheaths isolated with Sarkosyl (lane A) and SDS (lane B). Both lanes were loaded with 12 μg of protein. The three major bands visible in Sarkosyl-isolated sheaths are only faintly visible in the SDS-isolated sheaths. These three bands correspond closely to three of the major outer membrane protein bands reported for *L. discophora* SP-6 and SP-6(sl) (19).

defined; however, peptide cross-linking to form proteoglycans as discussed below is a strong possibility.

The protein component of the sheath must play an important role in the sheath structure. Indeed, disulfide bonds, presumably between sheath peptides, are required to maintain the structural integrity of the sheath fabric (20). Surprisingly, we were not able to identify major sheath structural protein(s) by gel electrophoresis. The major Coomassie blue-staining protein bands revealed by SDS-PAGE (Fig. 6) were found to be traces of contaminating outer membrane protein which were removed by SDS without alteration of the structure of the sheath. This suggests that the protein and polysaccharide components of the sheath may be covalently linked. It is possible that the 6.5-nm fibrils making up the sheath fabric could consist of a glycosylated core protein similar to the glycosaminoglycan chains of eukaryotic proteoglycans (5). Such proteoglycans have been described to exist in surface coat proteins of halophilic archaea (41). Glycosaminoglycans are common cell surface polymers of eukaryotes (5); and recently, a glycosaminoglycan was reported in a bacterial surface protein (42). Results of initial attempts at deglycosylating the sheath fibrils either enzymatically or chemically were equivocal (17). Thus, the nature of the protein-polysaccharide linkages in the structure of the sheath remains an open question.

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